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The use of low-cost brewery waste product for the production of surfactin as a natural microbial biocide

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ABSTRACT

Surfactin has potential as next generation antibiofilm agent to combat antimicrobial resistance against emerging pathogens. However, the widespread industrial applications of surfactin is hampered by its high production cost. In this work, surfactin was produced from *Bacillus subtilis* using a low-cost brewery waste as a carbon source. The strain produced 210.11 mg L^{-1} after 28 h. The antimicrobial activity was observed against all tested strains, achieving complete inhibition for *Pseudomonas aeruginosa*, at 500 μg mL^{-1}. A growth log reduction of 3.91 was achieved for *P. aeruginosa* while, *Staphylococcus aureus* and *Staphylococcus epidermidis* showed between 1 and 2 log reductions. In the anti-biofilm assays against *P. aeruginosa*, the co-incubation, anti-adhesive and disruption showed inhibition, where the greatest inhibition was observed in the co-incubation assay (79.80%). This study provides evidence that surfactin produced from a low-cost substrate can be a promising biocide due to its antimicrobial and anti-biofilm abilities against pathogens.

1. Introduction

Control of pathogenic microorganisms is essential for human health maintenance since they are responsible for several infectious diseases. Furthermore, the indiscriminate use of antibiotics in the past few decades has led to the selection of multiresistant microbial strains to traditional antibiotics, reducing the ability to treat diseases and enhancing the search for new compounds to improve the management of bacterial infections [1,2].

Bacteria colonization is often influenced by quorum sensing (QS), a mechanism, responsible for their ability to communicate with each other and to behave as a population. This phenomenon is fundamental for biofilm formation, in which the bacteria remains protected from environmental risks in a self-produced extracellular matrix, with high tolerance to chemical and physical treatments than planktonic cells forms [3,4].

The presence of biofilms may cause serious problems in the field of medicine and food industry. In hospitals, biofilms may result infections in patients with internal medical devices, such as urinary, endotracheal, intravenous, and other types of catheters and implants inserted into over 25% of patients during hospitalization [5,6]. In food industry, biofilms present on equipment or any related devices in direct contact with food will become a source of contamination, representing a risk to consumers through the transmission of diseases, as well as causing economic losses [7].

In this regard, the search for novel natural compounds with biocidal activity against pathogenic microorganisms is an urgent requirement. Biosurfactants are compounds capable of reducing the surface tension of liquid phases in contact with gas, or interfacial tension between immiscible liquids. The ability to reduce the surface/interfacial tension is due to the characteristics of the surfactant molecules, which have a hydrophilic and a hydrophobic moiety [8]. They can also interfere in biofilm development and communication between the cells and may cause rupture of membranes, causing cell lysis, and disruption of the surface properties affecting the adherence of the microorganisms [9].

Previous studies have shown the ability of different types of biosurfactants to disrupt and prevent biofilm formation. Elshikh et al. [10] reported treatment of oral pathogens with rhamnolipid showed a reduction of 3–4 log of bacterial viability. *Streptococcus*

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sanguinis showed 90% of biofilm inhibition when co-incubated with biosurfactant and 65% of biofilm disruption after treatment. In a similar investigation, Díaz De Rienzo et al. [11] reported the antimicrobial and biofilm disruption of *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006 using sophorolipids (5%, v/v).

Lipopeptides are the mostly widely known biosurfactants with antimicrobial activity, where surfactin produced by *Bacillus circulans* is the most prominent antimicrobial lipopeptide [12]. However, most studies reported in the literature present qualitative data, obtained mainly by using antimicrobial sensitivity tests (disk diffusion method). The mixture of lipopeptides (surfactin, iturin and fengycin) from *B. subtilis* showed significant anti-adhesive and antibiofilm activities on uropathogenic bacteria [13]. The effect of surfactin on adhesion and biofilm formation was evaluated by de Araujo et al. [7], in which the biosurfactant significantly reduced adhesion of *Pseudomonas fluorescens* ATCC 13525 on polystyrene surfaces (54% of inhibition) and a biofilm formation (73%) on stainless steel surfaces.

Considering, the properties of surfactin as antimicrobial and antibiofilm agent, there is much scope for their potential application in biomedicine. Although, the major hurdle in the widespread applications of surfactin is its high production cost. The production of biosurfactants depends on abundance and low substrate cost since it represents 30–50% of the value of the final product [14]. In relation to substrate costs, the use of agro-industrial waste in fermentation processes becomes an attractive alternative for the production of biosurfactants, since these residues often have favorable compounds for such processes, being a source of carbohydrates, protein, and micronutrients [14].

This work aimed to explore the application of biosurfactant, produced from a low-cost substrate, as antimicrobial and antibiofilm agent. *B. subtilis* ATCC 6051 produced biosurfactant using a brewery waste (Trub) as a substrate, where the chemical composition of the biosurfactant was investigated by MALDI-ToF-MS and FTIR analysis. The antimicrobial and anti-biofilm (co-incubation, anti-adhesive and disruption) activity of biosurfactant against several different pathogenic strains was further investigated. This work demonstrated the potential antimicrobial and antibiofilm of biosurfactant, being an interesting tool for food and medical field. In addition, it was possible to provide a suitable destination for the brewery waste, making the beer industry more competitive and contributing to the environment preservation.

2. Materials and methods

2.1. Microorganisms

B. subtilis ATCC 6051 was used for biosurfactant production and purchased from the Culture Collection of the Tropical Foundation for Research and Technology André Tosello (Campinas, SP, Brazil). *Pseudomonas aeruginosa* DSM 3227, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* DSM 20231 and *Staphylococcus epidermidis* DSM 28319 were used in the antimicrobial and antibiofilm assays.

The strains were stored in (1:1) Nutrient Broth medium (NB) with 20% glycerol at −80 °C. Whenever required, the frozen stocks of cells were streaked in plates with Nutrient Agar medium (NA) and incubated for 24 h at 37 °C. After incubation, the working stocks were kept at 4 °C.

2.2. Media composition and culture conditions of biosurfactant production

The carbon source used in the medium was Trub, which is a brewery waste resulting from the baking stage of the must and it was kindly provided by Kairós Brewery (Florianópolis, SC, Brazil). The Trub was characterized by Elemental Analysis and Enzymatic-Colorimetric Method. The results are shown in Table 1.

Biosurfactant production was carried out at 30 °C in a culture medium adapted from Maass et al. [15], composed of 2% (v/v) of Trub, 0.1 g L^{-1} of $CaCl_2$, 0.1 g L^{-1} of NaCl, 0.33 g L^{-1} of $FeSO_4 \cdot 7H_2O$, 0.0017 g L^{-1} of $MnSO_4 \cdot H_2O$, 1.69 g L^{-1} of KH_2PO_4, 0.50 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.90 g L^{-1} of peptone and 7.0 g L^{-1} of yeast extract. The pH of the medium was adjusted to 7.0 prior to sterilization at 121 °C for 20 min. The inoculum of *B. subtilis* ATCC 6051 was prepared by adding 2 mL of an overnight culture into 50 mL of NB, being subsequently incubated for 24 h at 30 °C. After incubation, the inoculum was standardized by adjusting its absorbance at 600 nm to 0.85 and added to the culture medium in a concentration of 5% (v/v). The flasks were incubated on a rotary shaker at 200 rpm for up to 28 h.

2.3. Recovery of biosurfactant

The biosurfactant was recovered by centrifuging (9000 rpm, 20 min) the culture broth in order to remove suspended solids (cells and solid particles from Trub). Subsequently, the pH of the supernatant was adjusted to 2.0 by adding HCl (4.0 M) and left overnight under refrigeration (4 °C) for precipitate formation [16–18]. The precipitated biosurfactant was centrifuged, washed twice with acidified water (pH 2.0) and resuspended in Milli-Q1 water (Millipore, USA). The pH of the solution was adjusted to 7.0, lyophilized, weighed and stored at −18 °C [15].

2.4. Determination of surface tension

A digital tensiometer (KSV, Sigma 702, Finland) was used for measure the surface tension of the cell-free supernatant by the Wilhelmy platinum plate method. Measurements were performed in triplicate at 25 °C. The critical micelle concentration (CMC) of the biosurfactant was determined by surface tension measurements of successive dilutions of aqueous biosurfactant solution, according to the methodology proposed by Sheppard and Mulligan [19].

2.5. Structural characterization of biosurfactant

The biosurfactant was chemically characterized by Matrix assisted laser desorption ionization time-of-flight mass

Table 1
Physico-chemical characterization of the Trub.

	Concentration $\left(g L^{-1}\right)$	Method
TOC	31.50	Total Organic Carbon Analyzer (TOC – SHIMADZU)
TC	31.91	
IC	0.41	
TN	3.45	Enzymatic-Colorimetric (Kit Gold Analisa Diagnostics)
	pH	5.73

TOC: total organic carbon, TC: total carbon, IC: inorganic carbon, TN: total nitrogen.

spectrometry (MALDI-ToF-MS), using a PerSeptive Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK) equipped with a 1 m time-of-flight tube. The system utilized a pulsed nitrogen laser set at 337 nm towards the densest area of the sample/matrix spot. The accelerating voltage was maintained at 20,000 V, the grid voltage and guide wire voltages were set at 93% and 0.05% respectively of the accelerating voltage. A solution of alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma Aldrich, UK) with a concentration of 10 mg mL^{-1} was prepared in 80% acetonitrile, 20% water with 0.1% trifluroacetic acid. 10 μL aliquot of sample was mixed with 10 μL of matrix and, subsequently, the samples were spotted on MALDI plate for analysis.

The functional groups and the chemical bonds present in the biosurfactant were analyzed using the Fourier transform infrared spectroscopy (FTIR) technique, using a Cary 660 Series FTIR Spectrometer – Agilent Technologies. For the analysis, a small sample of the dry bioproduct was mixed with potassium bromide (KBr). The FTIR spectrum was generated from 400–4000 cm^{-1}.

2.6. Investigation of the antimicrobial activity of biosurfactant

The antimicrobial activity of biosurfactant was carried out in 96-well microtiter plates (Sarstedt), in which different concentrations of biosurfactant dissolved in Mueller Hinton broth (MHB) were evaluated: *P. aeruginosa* (500-100 μg mL^{-1}), *S. aureus* (800–50 μg mL^{-1}), *E. coli* (800–50 μg mL^{-1}) and *S. epidermidis* (800–100 μg mL^{-1}).

In columns 1–10, 50 μL of biosurfactant in MHB were inoculated with 50 μL of selected microorganism, achieving a concentration per well of 5×10^5 CFU mL^{-1}. The inoculum was standardized by adjusting OD_{600} to a value corresponding to 10^8 CFU mL^{-1}. In the last two columns of the 96 well-plate, a growth control (broth with bacterial inoculum) and a sterility control (broth only) were established [20,21].

The well-plates were incubated at 37 °C for 24 h and serial dilutions were performed. 20 μL from the serial dilution of each biosurfactant concentration was dispensed in Mueller Hinton Agar (MHA) and incubated at 37 °C to obtain the CFU by Drop plate method [22,23]. All the concentrations were tested in triplicate and a $CFU_{Log_{10}}$ reduction after biosurfactant treatment were evaluated according to the equation:

$$\text{Log reduction} = \log_{10}A - \log_{10}B,$$

where *A* is the CFU mL^{-1} of growth control and *B* is the CFU mL^{-1} after biosurfactant treatment.

2.7. Investigation of the antibiofilm activity of biosurfactant

The biofilm formation ability of *P. aeruginosa* was evaluated according to O'Toole [24] and, in all the experiments, the absorbance of inoculum at 600 nm corresponded to a value equivalent to 10^8 CFU mL^{-1}. The tests were performed on polystyrene-24-well plate (Sarstedt) and untreated wells were used as controls.

The potential of the biosurfactant to prevent a biofilm formation was studied using two different techniques: co-incubation and anti-adhesive. In the co-incubation experiments, 1 mL of a range of biosurfactant concentrations (250–500 μg mL^{-1}) dissolved in NB was inoculated (5%, v/v) followed by incubation for 24 h at 37 °C. After the incubation time, the biofilm formed in Control wells and in the wells treated with surfactin were quantified by Crystal violet according to O'Toole [24]. This assay aimed to evaluated the inhibition of biofilm development due to the co-incubation of the strain with the biosurfactant.

The anti-adhesive activity was tested by pre-coating the wells with solutions of biosurfactant prepared in Phosphate buffered saline – PBS (250–500 μg mL^{-1}) in which 1 mL of each concentration was dispensed in the corresponding well and incubated for 24 h at 40 °C in order to improve the adsorption. After the adsorption time had elapsed, the plate contents were removed, and the wells were washed twice with PBS to removed unbound biosurfactant. The plate was sterilized for 3 h under UV light and 1 mL of standardized culture was added followed by incubation for 24 h at 37 °C.

The antibiofilm activity was also explored in the ability of the biosurfactant to disrupt an existing biofilm [25]. Initially, 1 mL of standardized culture was added in each well and the plate was incubated for 24 h at 37 °C for the biofilm development. After the incubation period, the planktonic cells were discarded, and the biofilm was washed twice with PBS. 1 mL of fresh media with different concentrations (200–700 μg mL^{-1}) of biosurfactant was added and incubated for 24 h at 37 °C.

At the end of all experiments, the plates contents were discarded, and the wells were washed twice with PBS to remove planktonic cells before staining the biomass attached to the surface of the wells. The plates were stained with crystal violet (0.1%) for 15 min at room temperature. Posteriorly, the plates were washed twice with sterilized water and left to dry overnight. One mL of acetic acid (30%, v/v) was added in each well and the absorbance of the content of the well was measured at 575 nm [24].

The percentage of the biofilm inhibition was calculated using the following equation,

$$\text{Biofilm inhibition}\ (\%) = 100 \times (OD_C - OD_t)/OD_C,$$

where OD_c and OD_t correspond to the optical density of the untreated biofilm and treated biofilm with biosurfactant, respectively.

2.8. Images

Scanning electron microscope (SEM) was employed to investigate the biofilm of *P. aeruginosa*. The control and biofilm assays (co-incubation, anti-adhesive and disruption) were carried out on coverslip as the adhering surface at 450 μg mL^{-1} surfactin concentration. At the end of each experiment, the coverslips were washed with PBS and immersed in 2.5% glutaraldehyde solution for 12 h. After that, the cells were dehydrated in graded ethanol (50%, 65%, 80%, 95% and 100% v/v) during 10 min and in hexamethyldisilazane (HMDS) in ratios of (1:1), (1:2), (1:3) and 100%, during 15 minutes each. The HMDS evaporated overnight and the samples were coated and analyzed under SEM [11].

2.9. Statistical analysis

The results were expressed as the mean ± SD (standard deviation) of 3 independent replicates. The data were analyzed using ANOVA (Analysis of variance), and the means were compared with the Duncan's test (5% probability). Significance of variances is indicated as follows: NS (Non-significant), $^{*}p < 0.05$, $^{**}p < 0.05$, $^{***}p < 0.005$.

3. Results and discussion

3.1. Biosurfactant production and characterization

Previous studies reported surfactin production using different medium composition, as mineral salt medium (MSM) with

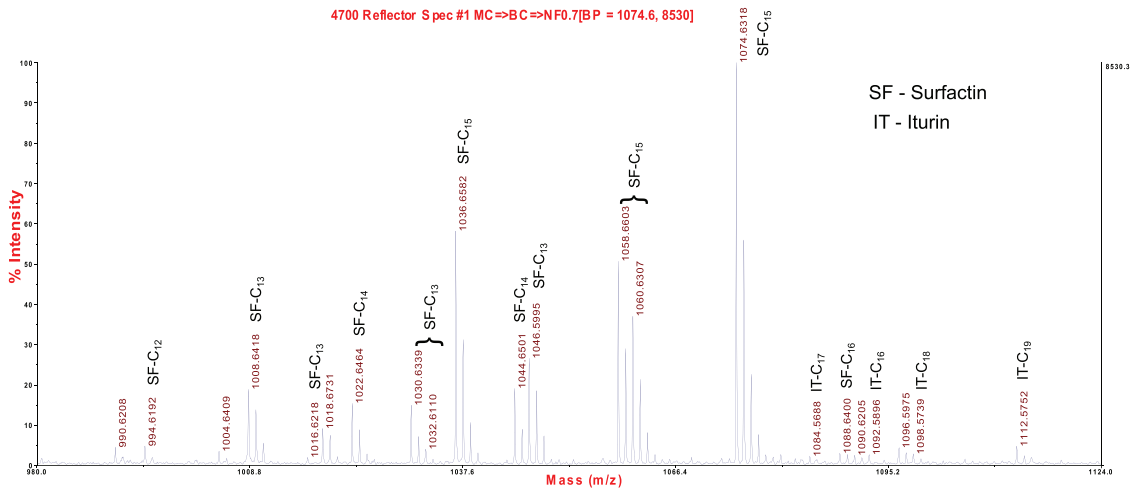

Fig. 1. MALDI-ToF-MS spectrum in positive mode of biosurfactant from *B. subtilis* ATCC 6051. The spectrum shown data for surfactin C_{16} (1088.6), surfactin C_{15} (1036.6, 1058.6, 1060.6, 1074.6), surfactin C_{14} (1022.6, 1044.6), surfactin C_{13} (1008.6, 1016.6, 1030.6, 1032.6, 1046.6) and surfactin C_{12} (994.6).

carbohydrate or nitrogen sources [26] and MSM plus trace elements [27]. However, due to their low yield and a high production cost, biosurfactant are unable to compete with the synthetic surfactant. Regarding this, a low cost production medium is important to minimize the production cost and achieve high yields. An alternative towards the commercialization of biosurfactants can be the use of cheap raw materials including agricultural and industrial wastes. For the first time, the production of surfactin was performed in culture medium using low-cost brewery residue (Trub) in a shake-flask fermentation. Within 28 h, the strain was able to reduce the surface tension of the culture medium from 54 to 28 mN m^{-1}. At the end of the batch, the surfactin concentration obtained was 210.11 ± 0.85 mg L^{-1}, confirming that Trub is a potential substrate for biosurfactant production.

Renewable resources have been used as carbon source in surfactin production as described by Ponte Rocha et al. [28], in which *B. subtilis* LAMI008 produced biosurfactant in mineral medium containing clarified cashew apple juice. When the medium was supplemented with yeast extract, the surfactin concentration was 3.5 mg L^{-1}.

Moya Ramírez et al. [29] evaluated surfactin production by *Bacillus sp.* using medium containing 2% (w/v) of Olive mil waste (OMW), achieving a maximum concentration of 3.12 mg L^{-1} after approximately 6 days of fermentation. The following year, the authors proposed a pre-treatment of the residue, proving that the enzymatic hydrolysis of OMW favored surfactin production, reaching a maximum concentration of 26.5 mg *textrmL*$^{-1}$ [30].

Efficient spectroscopic techniques have been utilized for investigations on the biochemical structure of biosurfactants, such as MALDI-ToF mass spectrometry and FTIR spectroscopy analysis. The MALDI-ToF mass spectrum revealed the presence of masses very similar to lipopeptide compounds, in which most of peaks could be attributed to the isoform of surfactins (Fig. 1). The peak at $m/z = 1074.6$ was clearly the most abundant in the spectrum followed by peak at $m/z = 1058.6$ and both correspond to potassium and sodium adducts of surfactin C_{15}, respectively. In addition, the following peaks with higher intensities refer to surfactin C_{13}, with mass numbers of $m/z = 1008.6$, 1030.6, 1046.6. The presence of surfactin C_{16} ($m/z = 1088.6$), surfactin C_{14} ($m/z = 1022.6$) and surfactin C_{12} ($m/z = 994.6$) was also verified. These results are in agreement with previous studies, since the authors also compared their results with standard surfactin [31–34].

Surfactin is able to disturb the membrane stability due to its interaction with the cell membrane [35]. According to Liu et al. [36], among the surfactin isorforms such as surfactin C_{13}, surfactin C_{14} and surfactin C_{15}, surfactin C_{15} was the most effective compound to interact with membranes due to the greater fatty acid chain inducing a greater interfacial activity of surfactins with the membrane. Iturin peaks, at negligible intensities, were also detected from the MALDI-Tof-MS (Fig. 1) analysis: Iturin C_{16} ($m/z = 1092.58$), Iturin C_{17} ($m/z = 1084.56$), Iturin C_{18} ($m/z = 1098.57$), and Iturin C_{19} ($m/z = 1112.57$) [31]. The concentration of Iturin was too low, suggesting it has no contribution to antimicrobial activity. In addition, no peaks of Fengycin were detected in the spectrum even Maldi-Tof-MS being a quite sensitive technique that allows detecting small amounts.

The FTIR spectra (Fig. 2) shows the lipopeptide nature of the biosurfactant since the main characteristic groups of surfactin molecule, such as aliphatic hydrocarbon and peptide-like moiety, are presented [37]. The peaks highlighted in Fig. 2 are the same presented for standard surfactin (Sigma–Aldrich, 98% purity), reported by Sousa et al. [37]. According to Joshi et al. [38], the bands at 3300–3400 cm^{-1} and 1650–1700 cm^{-1} (stretching mode of the CO—N bond) are characteristic of peptides. Absorbance in this region (3300–3400 cm^{-1}) occurred due to C—H and N—H

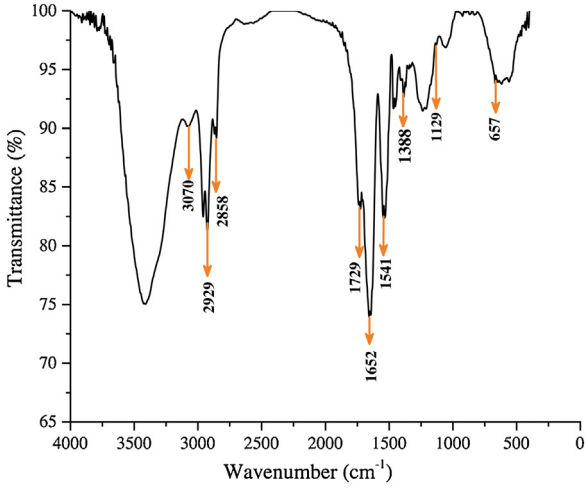

Fig. 2. Fourier transforms infrared spectrum of biosurfactants synthesized by *B. subtilis* ATCC 6051 using Trub as a carbon source.

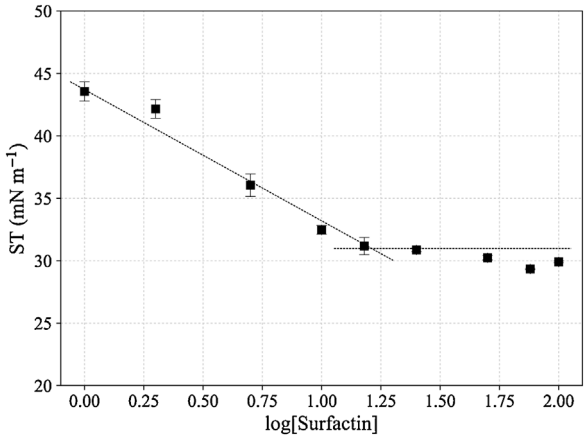

Fig. 3. The surface tension values of different concentrations of the surfactin. The intercept point represents the estimated CMC concentration.

stretching vibrations, been also characteristic of carbon-containing compounds with amino groups. Besides, the presence of intramolecular hydrogen bonding also corresponds to this region [12]. The aliphatic chains (—CH_3, —CH_2—) are exposed in the bands at 1200–1400 cm^{-1} [38] and the band at 1729 cm^{-1} is associated to the absorption of C=O groups from lactonization [37].

The critical micelle concentration (CMC) is an appropriate indicator of the biosurfactant efficiency since biosurfactants with low CMC values can be considered a good surfactant [39]. The CMC of the biosurfactant was determined by a plot of ST vs. log of surfactin concentration (Fig. 3), with a value of 15 mg L^{-1}. This value is close to those presented in the literature, in which Felix et al. [40] presented a CMC of 12.5 mg L^{-1} for surfactin synthesized by *B. subtilis* using clarified cashew apple juice as carbon source. In addition, the CMC reported in this work is also a satisfactory when compared to commercial standard surfactin (Sigma–Aldrich, 98% purity), whose CMC is between 7.5 and 20 mg L^{-1}, depending on methods.

3.2. Antimicrobial activity

The antimicrobial activity of the biosurfactant was tested against different microorganisms, which the highest inhibition for *P. aeruginosa* was achieved using 500 μg mL^{-1} of crude biosurfactant. At this concentration, the bacteria was completely killed. This concentration represents the minimum bactericidal concentration (MBC) which is the lowest concentration capable to eliminate a microorganism, i.e. not revivable under in a fresh sterile medium [12]. This phenomenon can also be confirmed by the ratio MBC/MIC, which is 2.5. To ratios ≤4.0, the agent can be considered bactericidal [41]. The MIC was determined as the minimum biosurfactant concentration that inhibited bacterial growth, which was 200 μg mL^{-1}. The treatment with 400 μg mL^{-1} of biosurfactant promoted a Log_{10} reduction of 3.91 ± 0.23 for *P. aeruginosa* (Fig. 4 (a)). This result shows the biocidal nature of

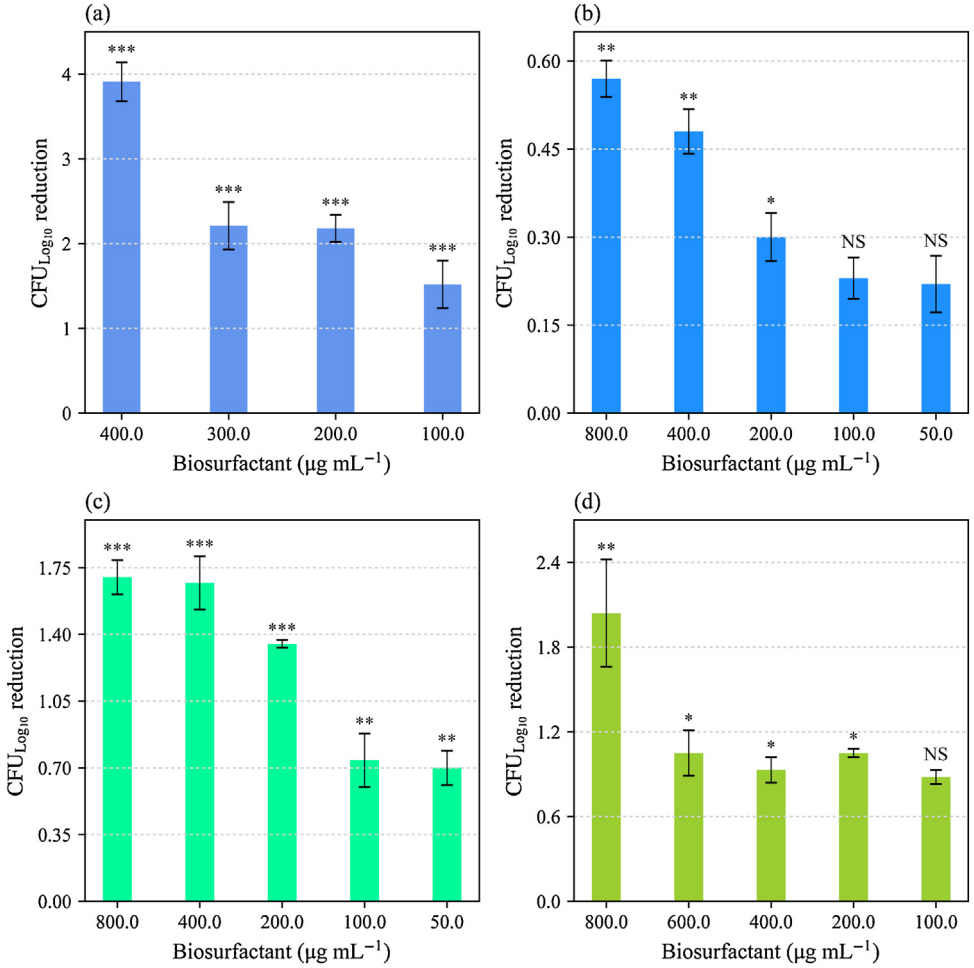

Fig. 4. Antimicrobial activity of different concentrations of biosurfactant (surfactin) against (a) *P. aeruginosa*; (b) *E. coli*; (c) *S. aureus* and (d) *S. epidermidis*. Values are represented as means ± SD ($n = 3$).

surfactin since it caused >3-log reduction as compared to the untreated bacteria after 24 h of treatment.

For the gram-positive strains, as *S. aureus* and *S. epidermidis*, the highest Log_{10} reduction using 800 μg mL^{-1} (Fig. 4c and d, respectively) were 1.70 ± 0.09 and 2.04 ± 0.38, respectively. The biosurfactant was less effective against *E. coli* with only 0.57 Log_{10} reduction at 800 μg mL^{-1} (Fig. 4b). These results were also in agreement with previous findings of Fanaei and Emtiazi [42], in which surfactin presented little or no activity against *E. coli* while showing inhibitory effect against *S. aureus* by disc diffusion method.

Santos da Silva et al. [43] evaluated the antimicrobial activity of surfactin against *S. aureus* ATCC-6533 and *E. coli* CCT-0355. The biosurfactant was produced by *Bacillus* sp. ITP-001 and the antimicrobial activity was determined by measuring zones of inhibition. While no antimicrobial effects were observed on *S. aureus*, a significant inhibition was detected against *E. coli* at 2300 mg L^{-1} [43]. In our study, we have used a microtiter plate assay to measure the antimicrobial activity. We could estimate that the difference in analysis methods could be one of the reasons for the difference in efficacy of surfactin to the previous report.

Sudarmono et al. [44] reported that surfactin produced by *B. amyloliquefaciens* presented antimicrobial activity against *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, showing Inhibition zone diameter of 20.0, 15.7 and 11.4 mm, respectively. The MIC was determined by resazurin assay in a 96 well-plate for *P. aeruginosa* (MIC > 1024 μg mL^{-1}). The different surfactin isoforms could be a reason for the higher MIC compare to this work since Sudarmono et al. [44] reported the presence of several isoforms of surfactin (C_{12}–C_{16}), mainly surfactin C_{16} and C_{12}.

The biocidal properties of surfactin observed in this work are very promising. The surfactin showed potential antimicrobial activity against both Gram-positive and Gram-negative bacteria. The lipopeptides from surfactin family are β-hydoxy hepta cyclic depsipeptides with possibilities of Ala, Val, Leu or Ile amino acid variations at positions 2, 4, and 7 in cyclic depsipeptide moiety and C_{13} to C_{16} variation in β-hydroxy fatty acid chains [32]. The variation in the structural composition of surfactin can be the main reason for antimicrobial activity, since it can significantly influence the physico-chemical properties and physiological activities, including interaction with the microbial membrane. The composition of surfactin is highly dependent on strain, culture condition, and growth medium composition [35,44].

Surfactins are mainly composed of C_{13}-surfactin, C_{14}-surfactin, and C_{15}-surfactin, and its amphiphilic structural contribute to interaction with cell membranes. The C_{15}-surfactin non-competitively inhibits the activity of the alkaline phosphatase due to the chelating action by the free carboxyl groups of the Asp and Glu residues. On the other hand, the binding of C_{15}-surfactin with lipopolysaccharides leads to the interruption of the lipopolysaccharides-induced pathway, inhibiting its activity [36].

Several studies in the literature evaluate the antimicrobial activity through the agar diffusion tests, which does not allow indicating values of concentrations of antimicrobial agents and their respective inhibitory effect on microorganism growth [43,45]. In our study, we carried out a throughput assay using 96-well plates, allowing us to evaluate the effect of different biosurfactant concentrations on the test microorganisms.

According to Kaczorek et al. [46], the biosurfactant acts in cellular phospholipid membrane, which is responsible for protecting their inner plasma membrane and cell wall from external toxic compounds [47], causing permeability due to the penetration of biosurfactant molecules through hydrophobic interactions. This permeability leads to a release of small metabolites, ions, enzymes, and supplementary substances from the cells and allows small molecules to enter cells and inhibiting their metabolism.

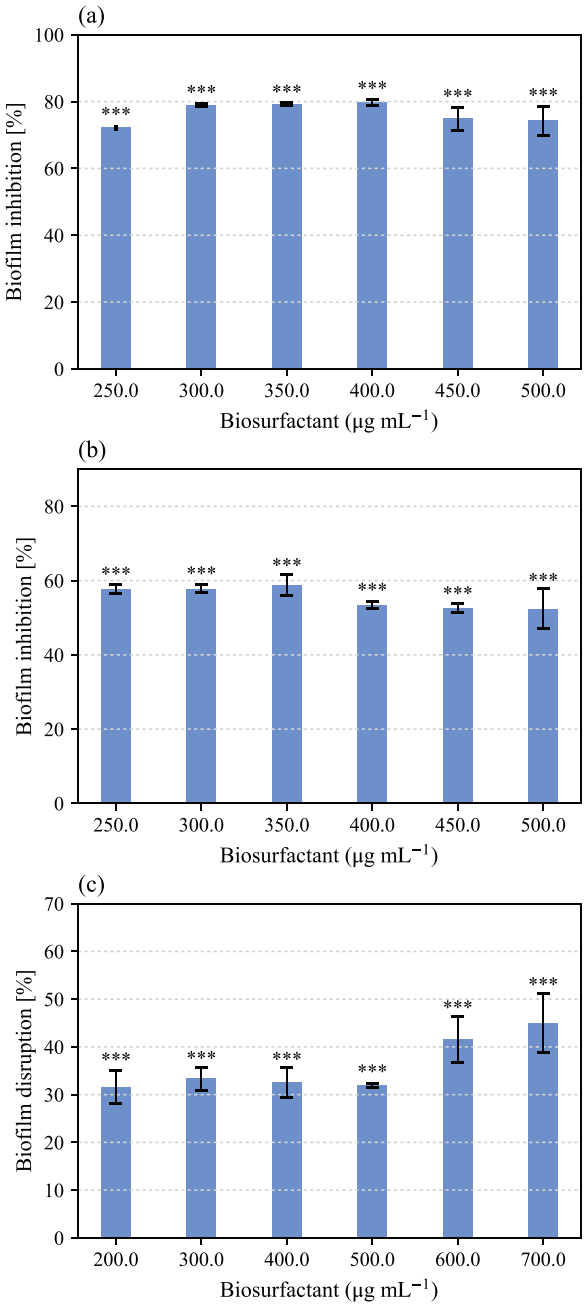

Fig. 5. Antibiofilm activity of biosurfactant (surfactin) against *P. aeruginosa* DSM 3227 at different concentrations: (a) co-incubation, (b) anti-adhesive and (c) disruption. Values are represented as means ± SD ($n = 3$).

Antimicrobial activity of surfactin is based on permeabilization of the cell membrane of the microorganisms due to the accumulation of biosurfactant on the microbial cell, causing its disintegration through the formation of pores in the cell membrane, inducing an increase in Ca^{2+} and H^{+} flux in the cells [48]. Moreover, the activity of surfactin is influenced by the concentration, since the biosurfactant is able to penetrate the membrane even at low concentrations, owing to the fact it is miscible with phospholipids, forming mixed micelles. At moderate concentrations of surfactin, the formation of ion-conducting pores in the membrane increases and, at high concentrations, the membrane is ruptured due to the detergency effect [49].

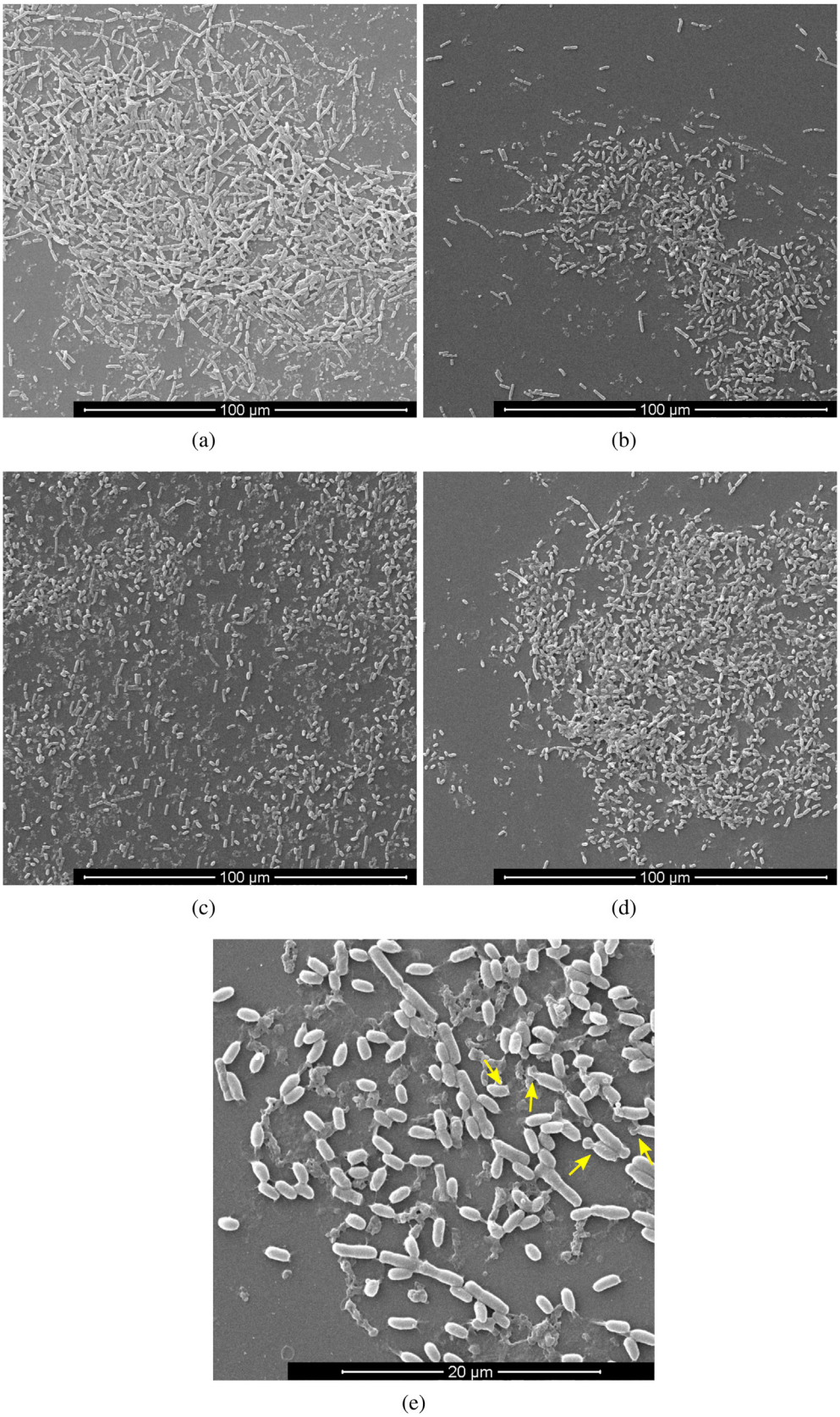

Fig. 6. Scanning electron microscopy images of *P. aeruginosa* DSM 3227. (a) Control shows biofilm formation after 24 h of incubation. Co-incubation (b), anti-adhesive (c) and disruption treatment (d and e). Damage in cells and outpouring of cell cytoplasmic is indicated with arrows. For all assays, 450 $\mu g\ mL^{-1}$ of biosurfactant were tested for 24 h.

3.3. Antibiofilm activity

The antibiofilm activity of crude surfactin was evaluated against *P. aeruginosa* and performed in three different ways: co-incubation, anti-adhesive and disruption. Optical density values were significantly different for the biofilm in the presence of the different treatments with respect to the control ($p < 0.005$).

The co-incubation assay was the most efficient, reducing biofilm formation by 79.80 ± .91% when using 400 μg mL^{-1} biosurfactant treatment. It is noted that percent inhibition remained between 72.14 ± 0.78 and 79.29 ± 0.89% for the other surfactin treatments and this inhibitory effect may have been caused by the antimicrobial activity of surfactin (Fig. 5a). Such inhibition of biofilm formation in co-incubation treatment has been described by Sriram et al. [50], who evaluated the antibiofilm activity of surfactin produced by *Bacillus cereus* NK1 against *P. aeruginosa*. This was the only work found by the authors, which reported surfactin antibiofilm activity when co-incubated with *P. aeruginosa*. The assay was performed in polystyrene 96 well-plate with Brain Heart Infusion broth (BHI) using 0.1–15 mg mL^{-1} biosurfactant concentrations. The highest percentage of inhibition reported was 54.21 ± 0.04% at 15 mg mL^{-1}. It is difficult to compare results in the literature, since fermentation for biosurfactants production provides a mixture of homologues, which can present different percentages of inhibition when used to inhibit biofilm of the same lineage of microorganisms [51].

To our knowledge, this study is the first reporting surfactin with anti-adhesion properties against *P. aeruginosa* biofilm. The anti-adhesive experiment revealed the highest inhibition of 58.81 ± 2.85% by pre-coating the surface with 350 μg mL^{-1} crude surfactin (Fig. 5b), suggesting that it is capable to modify the physico-chemical properties of the surface reducing adhesion and inhibiting biofilm formation. Moreover, lipopeptides alter the hydrophobicity of the bacterial surface and, consequently, alter the adhesion mechanism of the microorganisms. Its effects depend on the initial bacterial hydrophobicity, as well as the type of lipopeptide and its concentration, which may increase or decrease the hydrophobicity of the bacterial surface due to being more or less hydrophobic [52]. The anti-adhesive activity of biosurfactants has been described in previous reports, in which Janek et al. [53] present the ability of Pseudofactin II (0.5 μg mL^{-1}), a cyclic lipopeptide, to prevent biofilm formation on polystyrene surface of *E. coli*, *Enterococcus faecalis*, *Enterococcus hirae*, *S. epidermidis*, *Proteus mirabilis* and *Candida albicans*. Araujo et al. [7] observed that surfactin, at 0.50% (w/v), significantly reduced adhesion of *Listeria monocytogenes* on polystyrene surfaces when used at higher concentrations, reaching values of up to 54% inhibition. For, *Pseudomonas fluorescens*, the highest inhibition was only 17.1%.

In this study, the biosurfactant was also employed to disrupt a pre-existing biofilm, achieving 44.94 ± 6.19% of inhibition at surfactin concentration of 700 μg mL^{-1}. The percentage of inhibition remained similar for treatments between 200 and 500 μg mL^{-1} (Fig. 5c). The inhibition may have been induced by the removal of extracellular polymeric substances (EPS) and the destruction of microcolonies, caused by the biosurfactant [4]. The EPS plays an important role in the biofilm resistance, as it hinders contact of the microorganism with the antimicrobial agent [51]. Díaz De Rienzo et al. [4] pointed out that *P. aeruginosa* biofilms were disrupted by rhamnolipids at concentrations between 0.5 and 0.4 g L^{-1}.

3.4. Scanning electron microscopy (SEM)

SEM examinations of *P. aeruginosa* before (Fig. 6a) and after treatment were carried to visualize the effect of the surfactin (450 μg mL^{-1}) on biofilm formation and disruption. SEM analysis showed changes in biofilm morphology and topography as a result of treatment with the surfactin.

In the co-incubation assay, where cells were treated with surfactin for 24 h, the inhibition in biofilm formation was visible with changes in cell morphology observed. This effect was probably due to the antimicrobial activity of surfactin, which did not allow the full development of the biofilm. Furthermore, treated cells appear visually shorter than control cells (Fig. 6b). Similar observation was also reported by Dengle-Pulate et al. [54], in which *E. coli* cells had reduced size after treated with sophorolipids.

In the anti-adhesive test, the presence of free-living cells, in planktonic form was observed (Fig. 6c). However, they do not form a biofilm, suggesting that the coating with biosurfactant was effective in inhibiting biofilm formation. Similar observation was reported by Araujo et al. [7], who evaluated anti-adhesion activity of rhamnolipids (0.50%, w/v) against *P. fluorescens*, with a higher percentage of inhibition of 79%. The authors reported the growth of planktonic cells practically did not differ from the control, suggesting that biosurfactants do not affect planktonic growth and are adsorbed to polystyrene surfaces when used as surface conditioners.

In the rupture test, the biofilm was treated for 24 h with culture medium containing biosurfactant. After the treatment, regions of rupture within the biofilm were observed as well as the presence of cell in monolayers, while in the control the cells were distributed in multilayers (Fig. 6d). Damage in the cell membrane and outpouring of cellular cytoplasm after cell disruption is noticeable (Fig. 6e).

4. Conclusions

B. subtilis ATCC 6051 was able to grow in medium containing brewery residue and produce highly surface-active biosurfactant. Surfactin inhibited the growth of all microorganisms tested. The bactericidal effects were highest against *P. aeruginosa*. In addition, surfactin was also effective against *P. aeruginosa* biofilm, presenting the highest inhibition (79.80%) in the co-incubation assay, using a biosurfactant solution at 400 μg mL^{-1}. Therefore, the cost-effective production of surfactin together with antimicrobial and anti-biofilm activity makes it relevant for biomedical applications.

Authors' contribution

Talita Corrêa Nazareth: methodology, investigation, writing – original draft, data curation, validation. Conrado Planas Zanutto: writing – original draft. Lakshmi Tripathi and Abdulaziz Juma: investigation, data curation. Danielle Maass: conceptualization, writing – review & editing. Antônio Augusto Ulson de Souza, Selene Maria de Arruda Guelli Ulson de Souza and Ibrahim M. Banat: supervision.

Conflict of interest

None declared.

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